The lactose operon of Lactobacillus delbrueckii and its use for controlling gene transcription and/or expression in bacterial cells

The present invention relates to a DNA sequence suitable for controlled transcription and/or expression of a variety of different genes in bacteria, preferably in gram positive bacteria. In particular, the present invention pertains to a DNA sequence comprising the promoter/operator region of the lac operon of Lactobacillus delbrueckii with a DNA sequence coding for a gene product of interest being arranged in functional relationship therewith.

In the food industry a variety of different lactic acid bacteria are used to prepare food products, such as cheese, buttermilk or yogurt, essentially relying on the fermentative activity of said bacteria. For this purpose bacteria of e.g. the genus Streptococcus or Lactobacillus are put to use, with their primary function being to convert sugar, such as lactose or glucose, to lactic acid and to produce a satisfying texture and aroma.

A problem of any such fermentation resides in that the end product of the metabolic process, lactic acid, is often produced abundantly rendering the dairy product acid to an extent not accepted by most of the consumers. Thus, the persons attending the production process must take care so as to control the progress of the manufacturing process in order to avoid any detrimental effects of an excessive fermentation to the food product.

Lactic acid bacteria have also been used as fermenting agents for the preservation of food taking benefit of a low pH and the action of fermentation products generated during the fermentation process to inhibit the growth of spoilage bacteria. Also in such cases it is up to the personal to control the biological activity of the micro-organisms and to bring the production process at a desired stage to a stop.

Therefore, there is a need in the art to control the fermentative activity of micro-organisms used for the production of food, thus being capable to provide a fermentation up to a desired stage of the production process of the food product without involving too much labor work from the side of the attending staff.

In the recent past producers have tried to add valuable properties to bacteria utilized in fermentative processes via recombinant technologies. To this end, a vector bearing a homologous or heterologous gene coding for a polypeptide of interest is brought into the bacterial cell to either remain extra-chromosomal or to be inserted in the bacteria's chromosome. Expression is then effected by means of a heterologous promoter linked to the respective gene, which may be controlled as desired.

Though a control of the expression of the heterologous gene may be achieved thereby, this procedure is disadvantageous in that the regulon used to control the expression of the desired gene is in most cases exogeneous to the bacteria. Yet, the use of foreign gene material in bacteria utilized for the preparation of food products is presently not accepted by the consumer.

An object of the present invention is to provide means for a controlled trans-cription/expression of genes in bacteria that do not exhibit the disadvantages of the prior art techniques.

The above object has been solved by providing a DNA sequence exhibiting the following general formula

$$p/o - (A)_n - R_y, \text{ or}$$
$$p/o - R_y - (A)_n$$

wherein p/o denotes the DNA sequence identified under SEQ ID No. 9 or a functional variant thereof, which retains the capability to bind the lac repressor protein of Lacto-bacillus delbrueckii; A denotes a gene coding for a polypeptide of interest; n denotes an

integer of ≥ 0; R denotes the gene coding for the lacZ repressor protein as identified under SEQ ID No. 2 or a functional variant thereof; and Y is 0 or 1.

Consequently, in case $y = 0$ the gene coding for the lac repressor protein may be situated at any site of the bacteria's chromosome and may be subject to any suitable promoter region such that is e.g. expressed constitutively.

According to a preferred embodiment the DNA sequence is represented by the general formula $p/o - (A)_n - R$ or $p/o - R - (A)_n$, respectively, with p, A and R being as identified above. In this case, i.e. in the case of $Y = 1$, the construct is composed of three components, the promoter/operator region, the gene coding for the polypeptide of interest and the gene coding for the lac repressor protein (lacR), with the genes coding for the respective polypeptides (A, lacR) being arranged in either order. The lacR gene is such subject to its own promoter/operator. In addition, the gene coding for the lacR protein may be arranged such that its transcriptional direction is opposite to that of the gene A coding for the polypeptide of interest.

According to another preferred embodiment the gene coding for a polypeptide of interest is selected from group consisting of genes encoding enzymes, cell surface proteins or functional peptides, such as e.g. dextransucrase, glycosyltransferase, phytase, transgluta-minase, peptidase, phenylalanine ammonia lyase, protease, cell surface antigens, bacteriocins, hormones, insulin.

The promoter region may also lack any catabolite responsive elements, so that no repression in the presence of a particular carbon source may be effected.

According to another preferred embodiment the DNA sequence is used to transform a microorganism, which is preferably a gram positive bacterium or more preferably a microorganism selected from the group consisting of lactic acid bacteria, such as Lactococcus, Lactobacillus, Streptococcus, Leuconostoc etc..

The DNA sequence may be incorporated into the respective microorganism by means of an appropriate vector, such as a plasmid or a viral construct, and may remain in the microorganism extra-chromosomal. Yet, according to another preferred embodiment the construct may also be inserted into the bacteria's chromosome by means of conjugation followed by a crossing over between the plasmid and a homologous chromosomal sequence, or direct transformation and single or double cross over integration.

The DNA construct according to the present invention may therefore suitably be used for the transcription and/or expression of gene products, such as RNA or polypeptides.

In the figures,

Fig. 1 shows a comparison of L. delbrueckii promoter sequences;

Fig. 2 shows the organisation of the promoter region of L. delbrueckii LL44 and LB68:

Fig. 3 shows the organisation of the promoter region of L. delbrueckii N299;

Fig. 4 shows the nucleotide and amino acid sequence of the L. delbrueckii subsp. lactis LL44 lacR gene;

Fig. 5 shows a physical map of the lactose operon of different L. delbrueckii strains;

Fig. 6 shows a schematic representation of the construction of pLL 62;

Fig. 7 shows a schematic representation of the construction of pLL110 and pLL112 (CNCM I-2089);

Fig. 8 shows a schematic representation of the construction of pLL113, pLL115 (CNCM I-2090) and pLL116 (CNCM I-2091);

Fig. 9 shows the results of the expression of ß-glucuronidase using the constructs of the present invention;

Fig. 10 shows the results of experiments, wherein constructs of the present invention were used to express ß-galactosidase in L. delbrueckii.

The correct regulation of gene transcription and/or translation is a prerequisite for any cellular system to maintain its metabolic and catabolic turn-over and to respond to changes of the environment. In order to better adopt to environmental changes, such as a change of the nutritional source, microorganisms have developed various systems to control gene expression on both, the transcriptional and/or the translational level.

For the gram negative bacterium Escherichia coli several of such control systems have been studied. To this end, it has been found that the genes responsible for the catabolism of lactose, that is the genes coding for ß-galactosidase (lacZ), lactose permease (lacY) and transacetylase (lacA), respectively, are organized in a particular structural pattern which was termed operon. In this operon the genes coding for the structural polypeptides are located downstream a DNA region coordinating the transcription of said genes (operator) which region is negatively controlled by a repressor protein. The gene coding for the repressor protein is in turn controlled by a different promoter, which allows for a constitutive expression of the repressor.

Under repressive conditions the repressor protein binds to the promoter/operator region thus preventing transcription of the adjacent genes (lacZYA). Yet, in the presence of lactose or allolactose, which represent inducing compounds, the repressor forms a complex with the inducer, which complex has only a limited affinity to the promoter/operator, thus giving way for a transcription of the genes.

The regulation of the lac operon in gram positive bacteria, such as lactic acid bacteria, has been found to be also mediated via a polypeptide acting as a repressor, which is encoded by a gene termed lacR. This gene has been sequenced for Lactococcus lactis

(van Rooijen et al., J. Biol. Chemistry **265** (1990), 18499 - 18503) and Staphylococcus aureus (Oskouian et al., J. Bacteriol. **169** (1987), 5459 - 5465). Also in these microorganisms the repressor has a high affinity for the operator and binds to it in the absence of an inducer, resulting in a transcriptional blockade of genes located down-stream thereof. In the presence of an inducer molecule, such as lactose or galactose, the repressor is not capable to bind to the operon any more with the all structural genes following the operator region being transcribed together.

In lactic acid bacteria two different systems for the internalisation of lactose into the cells have been reported. Poolman et al. described in J. Bacteriol. **171** (1989), 244-253 a phosphoenolpyruvate dependent phosphotransferase system and a lactose permease system. It is reported that in Lactobacillus, Streptococcus and Leuconostoc lactose permease acts as a lactose/galactose antiporter system. The microorganisms internalize lactose from the medium as free sugar which is subsequently hydrolyzed to glucose and galactose. Galactose is either converted by the Leloir pathway into glucose-6-phosphate or is released into the medium by the permease.

In J. Bacteriol. **171** (1989), 244-253 and **173** (1991) 1951 – 1957, the cloning and se-quencing of the lactose permease genes (lacS) of S. thermophilus and L. delbrueckii, respectively, have been reported. The genes were found to be arranged in an operon structure each together with a gene coding for ß-galactosidase (lacZ). The gene for ß-galactosidase was disclosed for several lactic acid bacteria, such as S. thermophilus (Schroeder et al., J. Gen. Microbiol. **137** (1991), 369-380), L. bulgaricus (Schmidt et al., J. Bacteriol. **171** (1989), 625-635) and L. casei. (Chassy et al., FEMS Microbiol. Rev. **63** (1989), 157-166).

Yet, it has been found that the lac genes may also be subject to catabolite repression. In the presence of particular compounds, such as e.g. glucose, the genes of the lac operon are maintained in a repressed condition. This action is mediated by a cis acting element, that has been first described in Bacillus subtilis (Weickert et al., Proc. Natl. Acad. Sci. USA **87** (1990), 6238 - 6242) and has been termed "Catabolite Responsive Element"

(CRE). This element is present in a variety of gene arrangements encoding carbon catabolite enzymes in different gram positive microorganisms and seems to be controlled by the trans acting factors CcpA (Catabolite control protein A) and Hpr. In the presence of glucose both of these proteins bind to the CRE sequence acting as a negative regulation of transcription.

The species L. delbrueckii contains mainly two subspecies, L. lactis and L. bulgaricus, which are defined by several physiological and genetic criteria. One of these criteria concerns the regulation of the lactose (lac) operon expression. In the subspecies lactis, the expression is induced by lactose, that is the lac genes are expressed only in the presence of this sugar. In the subspecies bulgaricus, which has been selected a long time ago for its ability to ferment milk for yogurt production, the control of the lac genes expression was lost probably due to the constant availability of lactose in milk and the genes are constitutively expressed in the presence or in the absence of lactose.

For the experiments leading to the present invention six representative strains have been chosen to study the regulation of genes via the lac operon. Five strains belong to the subspecies lactis and have been termed LL44, LB68, N62 and N141, which are inducible by the presence of lactose, and LB10, that has been classified as belonging to the subspecies lactis but with a constitutive expression of the lac operon. The sixth strain, N299, corresponding to ATCC 11842, was chosen as a typical bulgaricus strain, with a constitutive expression of the lac genes.

During the analysis of the lactobacillus strains the region flanking the lactose (lac) operon was isolated and the following regions could be characterized.

The lac A gene

During the study of the promoter region of the lac operon, an open reading frame (ORF) of 570 bp was discovered upstream the lacS gene in the same orientation. This gene was completely sequenced for the L. delbrueckii subsp. lactis strain LL44 (SEQ ID. No 1) and it encodes a polypeptide of 190 amino acid residues (SEQ ID. No. 2). The presence of a

putative promoter and a potential ρ-independent-termination signal (stem-loop structure ; position 823, SEQ. ID. NO 1) suggest that this gene does not belong to the lac operon.

The promoter region

The region comprised between the end of lacA and the beginning of lacS (lactose permease) was sequenced and showed several attributes specific for a promoter. Two palindromic sequences, called O1 and O2 (Fig. 1), were discovered and may serve as operators for the binding of the lac repressor (LacR). The operators of the 6 strains studied (LL44, LB68, N62, N141, LB10 and N299) were sequenced. Several differences were found, which were partly due to the insertion of IS-elements in the O1 inverted repeats and partly due to small nucleotides changes in the sequence (Fig. 1). ISL7 is inserted in N141 exactly at the TGT motif of the 5' end of O1, but restoring the bases TG (Fig. 1). In N299, the L. delbrueckii subsp. bulgaricus strain, the insertion of ISL5 at the same position destroyed the TGT motif, which might explain the constitutive transcription of adjacent genes.

In lactic acid bacteria, effective repression of transcription is deemed to involve the cooperative binding of LacR to two different operator sequences. In L. delbrueckii, a small operator, called O2, was found 4 bp downstream O1. The length of O2 showed considerable variation in length between the 6 strains analysed due to small nucleotide changes in the sequence (Fig. 1). However, no IS-element was found in this operator. The core of both inverted repeats is composed of the nucleotides TGTTTA (SEQ. ID. No. 3) and (SEQ. ID. No. 4), except in LL44 where the final A was replaced by G in O2 (SEQ. ID. No. 5).

A sequence of 14 nucleotides homologous to the catabolite responsive element (CRE) (Weickert, supra), was discovered 40 nucleotides upstream the lacS start codon of all the L. delbrueckii strains studied (Fig. 2, Fig. 3, SEQ ID No. 8)). Catabolite repression is effective in many bacteria and acts at the level of transcription via negative transcriptional control. It involves a cis-acting element mediating repression of the genes under its control in the presence of glucose. The sequence of these elements is highly conserved among

different species and the one of L. delbrueckii subsp. lactis is highly similar to other elements.

The figures 2 and 3 present a view of the promoter region of the lac operon of 5 of the strains studied. They are considered as representative of the different possible lac operon organisations in the L. delbrueckii species. Four inducible lactis strains, LL44, LB68, N62 and N141 and one constitutive bulgaricus strain, N299 were represented. Strain LB10 was not shown, because its promoter region is identical to N62 except that ISL6 is not present in the lac promoter of this strain. The initiation of transcription in N299 (Leong-Morgenthaler et al., J. Bacteriol. 173 (1991), 1951-1957) falls in the middle of the CRE element, just after the three nucleotides change that were found in the CRE element of this strain (Figs. 2 and 3).

LL44 was chosen as a reference due to the absence of any IS-element in its lac operon region. In this strain, the lacA gene is followed by the two operators O1 and O2 and the CRE element upstream the lacS gene. In other strains, the presence of IS-elements changed the promoter sequence and particularly the O1 operator sequence.

The lac R gene

Surprisingly, and in contrast to the gene arrangements of known lac operons the gene coding for the repressor protein was discovered downstream the lacZ gene in L. delbrueckii subsp. lactis LL44 and LB68, with the result that the repressor is under its own transcriptional control.

The region covering the lacR gene was PCR amplified using the following primers:

CGCCTGGTGATTCAGCC   (SEQ ID No. 6)

AGCTTTACGGGGAAGTCGGG   (SEQ ID No. 7)

which are located at the end of the ß-galactosidase (lacZ) gene (SEQ ID No. 6) and in the Asn-tRNA synthetase (asnA) gene (SEQ ID No. 7).

Sequencing of this region revealed the sequence identified under SEQ ID No. 8, which exhibits an open reading frame of 999 bp in the same orientation as lacZ. The lacR gene is preceded by a ribosome binding site (RBS) and followed by a putative ρ-independent-termination signal (stem-loop structure ; position 1149). The putative amino polypeptide derived therefrom is shown under SEQ ID No. 2 (333 amino acid residues).

Computer-assisted analysis of the L. delbrueckii lacR gene predicted the protein secondary structure of the beginning of the gene (positions 4 to 23) to be a helix-turn-helix motif, which represents the highest homology region with other repressors. This type of protein secondary structure is a common feature for DNA-binding proteins and binds to the operators located in the promoter region.

The lac operon

The organisation of the lac operon of different L. delbrueckii strains is represented in figure 5. The lac operon per se is preceded by the thiogalactosyl-transacetylase (lacA) gene, which is followed by a rho-independent termination signal. This gene is followed by the promoter region of the lac operon, which comprises the inverted repeats (operators) involved in the regulation of the gene expression and the CRE sequence. The lac operon is composed of three genes, the permease (lacS), the ß-galactosidase (lacZ) and the repressor (lacR). The lacS and lacZ genes are separated by 4 nucleotides, whereas lacR is distant from lacZ by 52 nucleotides.

Five IS-elements have been found in the promoter and the terminator region of the lac operon, which were called ISL3, ISL4, ISL5, ISL6 and ISL7. One of these Elements, ISL3, has been described in Germond et al, Mol. Gen. Genet. 248 (1995), 407-416.

The lactose permease (lacS), the ß-galactosidase (lacZ) and the repressor (lacR) constitute the lac operon per se. The three genes are linked together as a lacSZR operon in L. delbrueckii subsp. lactis without any promoter in between. This gene arrangement is not a common feature for regulated operons, as the lacR gene is normally not a part of the operon.

The lac operon of L. delbrueckii is furthermore induced exclusively by lactose, and, as part of the operon, the repressor is deemed to be increasingly produced under inducible conditions. In L. delbrueckii subsp. bulgaricus, the typical yogurt strain, the lacR gene was inactivated by small nucleotides insertions and deletions in the sequence resulting in the constitutive phenotype of this subspecies.

In L. delbrueckii subsp. lactis, the repressor is able to bind to the promoter region which is located upstream the lacS gene. This region contains two inverted repeated sequences, O1 and O2, which seem to assist in the binding. The presence of two operators stabilizes the complex with the repressor in vitro, which occurs via DNA looping between the operator and the different subunits of the repressor.

The degree of repression depends from the position of the operators in the promoter region. A repressor bound between the –10 element and the start codon suffers in competition with the polymerase since this molecule can form a complex with the –35 element, whereas an operator located upstream the –35 element is not very effective since it allows the polymerase to access the –10 element and the initiation site. An operator located in the spacer region ensures a tighter control of the operon. The O1 operator of L. delbrueckii is located upstream the –35 element, whereas the O2 operator is located in the spacer region. This operator is very variable in length, ranging from 11 nucleotides in LL44 to 22 in N299.

To study the functionality of the promoter/repressor system in vitro, the promoters of the inducible strain LL44 and constitutive strain N299 were cloned in front of reporter genes, e.g. chloramphenicol acetyltransferase or ß-glucuronidase, in the presence or absence of the repressor gene. Different expression systems were tried in E. coli and Lactococcus lactis.

Induction of ß-glucuronidase production showed considerable differences between the different promoters associated to LL44 lacR. The plasmid carrying the LL44 promoter (pLL112) was fully induced by 1.0% lactose up to levels of 250 mU /mg protein, whereas plasmids carrying the N299 promoter (pLL116) reached about 100 mU/mg protein when the lacR gene was in the reverse orientation as the gusA gene. When the lacR gene was

introduced in the same orientation as the gusA gene (pLL115), levels of induction dropped to around 40 mU/mg protein. This may be explained by the absence of a promoter in front of lacR and its control either by the N299 strong lac promoter when it is in the sense of the gusA gene or by a weak promoter which must be located on pNZ272 when it is orientated antisense. It was also not possible to obtain the lacR gene in the same sense than gusA with the LL44 promoter. Production of GusA also showed that all plasmids were subjected to glucose catabolite repression due to the presence of a CRE sequence in the promoter of L. delbrueckii. The CRE of N299 is partly disrupted and the repression observed with this promoter is weaker than with LL44.

Measurements of the ß-galactosidase activity in vivo revealed that the L. delbrueckii strains were almost not subjected to glucose catabolite repression. The β-galactosidase activity also confirmed the inducible characteristics of the L. delbrueckii subsp. lactis strains and the constitutivity of the bulgaricus strains. Indeed, LL44, LB68, N62 and N141 were not induced in the presence of glucose as sole carbon source, whereas in N299 β-galactosidase activity was also high in the glucose medium. The inducible characteristic of an operon allows the strain to quickly switch from metabolizing one substrate to another.

Thus, according to the present invention the transcription and/or expression of homologous and/or heterologous genes in bacteria may be suitably obtained by preparing the construct p/o-$(A)_n$-R_y or p/o-R_y-$(A)_n$ as illustrated above and introducing said construct into the bacterium.

According to the present invention it is now possible to control the transcription and/or expression of a specific gene polypeptide by adjusting the content of glucose and/or lactose, respectively, in the culture medium. As an example, the bacterium may be grown in a medium lacking lactose to a desired degree and in a next step may then be transferred to a medium containing lactose or lactose is simply added to the medium. Only at his stage the repressive activity of the construct on the transcription and/or expression of the gene of interest will be relieved and the gene product will be produced.

In order to assist the inductive activity of the construct the gene for the lactose permease may be one of the polypeptides designated "A". To this end, if an amount of lactose enters the bacterial cell the genes following the promoter described here are transcribed with one of the polypeptides to be expressed thereby being lactose permease, which actively transfers additional lactose into the cell.

Further, it may well be suitable to delete the region harboring the catabolite responsive element, so that the construct is not subject to carbon repression.

The following examples illustrate the invention without limiting the scope of the appended claims.

Example 1

Bacterial strains and growth conditions

Lactobacilli were grown in Difco Lactobacillus MRS-broth (DeMan et al., J. Appl. Bacteriol. **23** (1960), 130-135) or in Oxoid BHI-broth. Lactococci strains were cultivated in Difco M17-broth (Terzaghi et al., Appl. Microbiol. **29** (1975), 807-813) supplemented with 0.5% glucose (GM17). E. coli was grown in YT-broth 2x prepared according to Maniatis et al., Molecular cloning : A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). All strains were stored at -80°C in their respective media containing 10% glycerol ten times concentrated.

For plasmid selection and maintenance in Escherichia coli, ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), or kanamycin (50 μg/ml) were added to the growth medium (ampicillin and kanamycin obtained from Sigma, chloramphenicol from Boehringer Mannheim). For plasmid selection and maintenance in Lactococcus lactis MG1363, chloramphenicol at 12 μg/ml was added to the growth medium.

Chromosomal and plasmid DNA purification

Lactobacilli DNA was prepared according to Delley et al., Appl. Environ. Microbiol. **56** (1990), 1967-1970. Plasmid DNA from E. coli was purified by the alkali lysis method (Maniatis, supra). Lactococcus plasmid DNA was prepared following the same procedure,

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except that the cultures were first incubated 30 min at 37°C in TE (10 mM Tris-HCl, pH 8.0 ; 1 mM EDTA) containing 10 mg/ml lysozyme (all chemicals obtained from SIGMA).

DNA manipulations and bacterial transformations

Agarose gel electrophoresis, restriction enzyme digestions, ligations and transformations in E. coli were performed according to standard procedures (Maniatis, supra). When necessary, the digested DNA was blunt-ended with T_4 DNA polymerase (Boehringer-Mannheim) directly in the digestion mixture for 5 min at 37°C.

The different L. delbrueckii operators were cloned into plasmid pKK232-8 (Ap^r, Cam^r, contains a promoterless chloramphenicol acetyltranferase (cat) gene, E.coli vector, Pharmacia) and transformed in E. coli SURE (obtained from STRATAGENE). Transformants were selected on YT-agar plates containing 30 µg/ml chloramphenicol. The LL44 lacR gene was cloned into plasmid pKK223-3 (Ap^r, contains a strong tac promoter, Pharmacia) and transformed in E. coli SURE. Transformants were selected on YT-agar plates containing 100 µg/ml ampicillin. For lacR overexpression, 2 mM isopropyl-β-D-thiogalactoside (IPTG) was added to the culture and cells were allowed to grow for an additional 4-5 hours at 37°C. Cultures were centrifuged and the pellets were resuspended in 0.1 M Hepes, sonicated and centrifuged. Supernatants were applied to a SDS/PAGE gel (Laemmli, Nature 227 (1970), 680-685).

When the lacR gene was cloned into plasmid pET11c (obtained from STRATAGENE), plasmids were transformed in E. coli BL21 (STRATAGENE). Transformants were selected on YT-agar plates containing 100 µg/ml ampicillin, and lacR was overexpressed as described above in the presence of 1 mM IPTG. The lacR gene was finally cloned into plasmid pACYC177 (New England Biolabs) into the PstI site of the ampicillin gene and transformed in E. coli XL1-Blue (SRATAGENE). Transformants were selected on YT-agar plates containing 50 µg/ml kanamycin. Plasmid pACYC177 containing the lacR gene was cotransformed with the pKK232-8 plasmids containing the different operators in E. coli XL1-Blue. Transformants were selected on 100 µg/ml ampicillin for the presence of pKK232-8 and 50 µg/ml of kanamycin for the presence of pACYC177.

Competent cells and transformations of Lactococcus lactis MG 1363 (plasmid free deriva-tive of NCDO 712; Gasson, M.J., NCDO (1983) 712) and other lactic streptococci after protoplast-induced curing, J. Bacteriol. 154:1-9) were realized according to Holo et al. Appl. Environ. Microbiol. 55 (1989), 3119-3123. Cells were grown in GM17-broth containing 500 mM sucrose and 3% glycine to an OD_{600} of 0.2 - 0.3, centrifuged and washed several times in a solution containing 500 mM sucrose and 10% glycerol. Competent cells were stored 100x concentrated at -80°C in the same solution until use.

Cells (40 µl) were mixed with 5 µl of plasmid DNA and electrotransformed at 2.0 kV, 25 µF and 200 Ω. Cells were resuspended in GM17-broth containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and expressed for 90 min at 30°C. Cells were then plated on GM17-agar containing 12 µg/ml of chloramphenicol. When the different L. delbrueckii promoters were cloned upstream the GUS gene, 50 µg/ml of X-glu (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) (Amresco) was added to the GM17-agar plates to select for blue colonies.

DNA amplification and sequencing

DNA amplification was done by polymerase chain reaction (PCR) in the presence of 0.2 mM of each dNTPs, 1 µM of each oligonucleotide primers, the appropriate buffer and the Taq polymerase (Gold polymerase, Perkin-Elmer) under the following conditions : 1 min at 94°C - 2 min at 55°C and 3 min 30 at 72°C for 35 cycles. Custom made oligonucleotide primers were used. DNA sequences were determined directly by PCR using the VISTRA Thermo sequenase sequencing kit (Amersham) (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467) and then analysed with the help of the University of Wisconsin, USA, Genetics Computer Group (GCG) computer software package (Devereux, Nucleic Acids. Res. 12 (1984), 387-395).

Isolation of spontaneous repressor negative LL44 mutants

The L. delbrueckii subsp. lactis strain LL44 was isolated on MRS-agar containing glucose and 200 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) (Sigma) to check the inducible state of its lac operon. One white colony was further incubated overnight at 42°C in MRS-broth containing 10% lactose. The culture was stored refrigerated for 24 hours and then subcultured in MRS-broth containing 10% lactose for 7h

at 42°C. This culture was diluted and plated on Oxoid Reinforced Clostridial Medium (RCM) agar containing only glucose and 200 µg/ml X-gal to select for a blue lac constitutive mutant. Plates were incubated for 72 h at 40°C under microaerophilic conditions (BBL, microaerophilic system No. 71034). A blue colony was obtained, further cultivated and stored in MRS-broth as described above.

Chloramphenicol acetyltransferase assay (Shaw, Methods Enzymol. 43 (1975), 737-755)

Cells were grown overnight in YT-broth 2x containing the necessary antibiotics (see DNA manipulations and bacterial transformations) at 37°C under shaking. 10 ml of fresh YT-broth containing either 0.5% lactose or 0.5% glucose was inoculated with 1% of the overnight culture. Kanamycin (50µg/ml) and/or ampicillin (100 µg/ml) were used as antibiotics. Cells were incubated for 3 hours at 37°C, centrifuged and the pellet was resuspended in 2 ml Hepes 0.1 M. The suspension was sonicated for 30 sec, spun down and the supernatant was mixed with 1 volume of buffer 2x concentrated (200 mM Tris-HCl, [pH 8.0] – 0.2 mM Acetyl CoA – 0.8 mg/ml DTNB) and chloramphenicol was added at a final concentration of 0.1 mM. The mixture was incubated at 37°C for 4 min and the OD red at 412 nm.

In a second cycle of experiments, the YT-broth was replaced by a solution containing : 1/5 vol M9-salts 5x ($Na_2HPO_4 \cdot 7H_2O$, 450 mM – KH_2PO_4, 110 mM – NaCl, 45 mM – NH_4Cl, 93 mM), 0.5 % glucose or lactose, 1 mM $CaCl_2$, 20 mM $MgCl_2$ and 10 mM thiamine. The rest of the assay was realised as described above.

Determination of ß-glucuronidase activity

(modified from Bergmeyer, Methods of Enzymatic Analysis 2 1983, 206-209)

Starters of transformed Lactococcus lactis were prepared by incubating the bacteria overnight at 30°C in M17-broth containing 0.5% mannose and 12 µg/ml chloramphenicol. Ten ml of M17 containing different sugars were inoculated at 2% with the overnight culture and incubated at 30°C to an OD_{660} of about 1.0. Following centrifugation, cells were stored at -20°C until use. For the assay, they were resuspended in 2 ml of distilled water containing 0.1% Triton X100, incubated 30 min at room temperature and collected by centrifugation. Cells were then resuspended in 2 ml of GUS buffer (50mM $NaHPO_4$ [pH 7.0], 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X100) containing 50

μg/ml of para-nitrophenyl-β-D-glucuronide (Clonetech). After 1 hour incubation at 37°C, 500 μl were mixed with 500 μl of stop solution (2 mM Na_2CO_3) Centrifugation was followed by absorbance measurement at 415 nm. ß-glucuronidase activity was calculated by using the equation :

$$U/mg\ prot. = OD_{415} \times dilution\ factor/18 \times t \times mg\ prot./ml$$

wherein t is the reaction time, 18 the millimolar extinction coefficient of the para-nitrophenol and 0.4 the dilution factor.

The protein content of the culture was determined as described by Bradford. Anal. Biochem. **12** (1976), 248-254, using the Bio-Rad protein assay with bovine serum albumin as standard. Samples of 0.1 ml of the culture were mixed with 5 ml Bio-Rad dye reagent (diluted 1:4 with distilled water). After incubation for 5 min, the absorbance was red at 550 nm.

For the ß-glucuronidase induction, fresh M17-broth containing 0.5% mannose and 12 μg/ml chloramphenicol was inoculated at 2% with the starter cultures and incubated at 30°C to an OD_{660} of about 0.2. The cultures were split and the different sugars added. Cultures were further grown at 30°C until the OD_{660} reached 1.0. ß-glucuronidase activity was measured as described above at 60 min intervals.

Determination of ß-galactosidase activity (modified from Bergmeyer, supra)

Starters of L. delbrueckii were prepared by incubating bacteria overnight in MRS-broth at 42°C. 10 ml of BHI containing different sugars (table I) were inoculated at 2% with the overnight culture and incubated at 42°C to an OD_{600} of about 1.0. For the Lactobacillus delbrueckii subsp. lactis strain N141, galactose was replaced by mannose (as galactose induced the lac operon). Following centrifugation, cells were stored at -20°C until use. For the assay, pellets were resuspended in 2 ml of distilled water containing 0.1% Triton X100, incubated at room temperature for 30 min, and collected by centrifugation. Cells were resuspended in 2 ml of Z buffer (0.06 M Na_2PO_4 ; 0.04 M $NaH_2PO_4 \bullet 7H_2O$; 0.01 M KCl ; 0.001 M $MgSO_4.7H_2O$; 0.05 M β-mercaptoethanol [pH 7.0]) (Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Sring Harbor, N.Y., 1972),

diluted 10 times in the same buffer containing 50 µg/ml ortho-nitrophenyl-β-D-galactopyranoside (ONPG). After 30 min incubation at 42°C, 200 µl were mixed with 800 µl of stop solution (400 mM Na_2CO_3; 50 mM EDTA) Centrifugation was followed by absorbance measurement at 420 nm. ß-galactosidase activity was calculated by using the equation :

$$U/OD_{600} = OD_{420} \text{ x dilution factor} / 3.5 \text{ x t x } OD_{600},$$

wherein t is the reaction time, 3.5 the millimolar extinction coefficient of the ortho-nitrophenol and 10 the dilution factor.

For the induction of ß-galactosidase, fresh BHI-broth containing 0.5% galactose was inoculated with the starter cultures and incubated at 42°C to an OD_{600} of about 0.2. The cultures were split and the different sugars were added. Cultures were further grown at 42°C to an OD_{600} of about 1.0. ß-galactosidase activity was measured as described above at 15 min intervals.

Example 2

Expression of the L. delbrueckii LL44 lacR gene in E. coli

The lac repressor (lacR) gene of L. delbrueckii strain LL44 was tentatively expressed in E. coli. It was first PCR amplified using the following primers

Pst I
ATATTACTGCAGAGTAAAAGCGAGT   SEQ ID No. 10

Hind III
ATAAATAAGCTTACAGAATGCAGCC   SEQ ID No. 11

which contain a PstI and a HindIII site, respectively. The fragment was cloned in the corresponding sites behind the strong tac promoter of pKK223-3, a high copy-number plasmid, and transformed in E. coli. The presence of lacR in the resulting clone, termed pLL56, was verified by sequencing. Upon 2mM IPTG induction an expression of the gene could be obtained.

Example 3

Cloning of the L. delbrueckii lac promoter in front of the chloramphenicol acetyltransferase gene in Escherichia coli

The lac promoters of L. delbrueckii LL44, N141 and N299 were tentatively cloned in front of an E. coli reporter gene, the chloramphenicol acetyltransferase (cat) gene. They were PCR amplified using the following primers

```
                    Eco RI
        ATATTAGAATTCAGTGACTTAAACTGG        SEQ ID No. 12


                    Eco RI
        ATATTAGAATTCAGTACTTTGACACCG        SEQ ID No. 13


                    Eco RI
        ATATTAGAATTCAAGAGGCTATATCGC        SEQ ID No. 14


        GGTTAATGCCGCCAAAGT                 SEQ ID No. 15
```

which contain an EcoRI site and primer 250 which is located in the lacS gene. The amplified fragments were digested with EcoRI and BspEI, which cleave in the primer sequence and 170 bp downstream the start codon of lacS. Restriction sites were filled with T_4 DNA polymerase, cloned in front of the promoterless cat gene of pKK232-8 digested with SmaI and transformed in E. coli. Chloramphenicol resistant clones were obtained for the three lactobacilli lac promoters, which indicates that the lac promoters of L. delbrueckii are active in E. coli. They were called pLL55 for N299, pLL57 for N141 and pLL58 for LL44 (Table 2) and all the constructs were verified by sequencing.

Example 4

Control of the L. delbrueckii lac promoters by the LL44 lacR gene in E. coli

To test if the L. delbrueckii lac promoters could be controlled by the LL44 lacR in E. coli, plasmids pLL55, pLL57 and pLL58 were cotransformed with a low copy number plasmid containing the LL44 lacR gene under the control of the promoter of E. coli lac repressor (lacI). To obtain this construct, the two DNA fragments were linked by the Gene Splicing

by Overlap Extension (gene SOEing) method (Horton R., Molecular Biotechnology <u>3</u> (1995), 93 – 99).

The promoter of lacI was first amplified from pET11c using the following primers

<div align="center">

Pst I

ATAAAT<u>CTGCAG</u>TGGGTATGGTGGC   SEQ ID No. 16

GATCGTTGCCACATTCACCACC   SEQ ID No. 17

</div>

The primer SEQ ID No. 18 is composed of a sequence of the lacI promoter and of the L. delbrueckii 5' end of lacR. The complete lacR gene was then PCR amplified using the following primers

<div align="center">

GGTGAATGTGGCAACGATCAG   SEQ ID No. 18

Pst I

ATATTA<u>CTGCAG</u>ACAGAATGCAGCC   SEQ ID No. 19

</div>

The sequence of primer SEQ ID No. 19 is the complement of SEQ ID No. 20. Both PCRs were purified, mixed and reamplified with primers SEQ ID No. 16 and SEQ ID No. 19, linking the lacI promoter and the LL44 lacR gene. Additionally, the ATG start codon of the lacR gene was replaced by GTG as in lacI (Fig. 6). The whole construct was cloned in the PstI site of the low copy number plasmid pACYC177 (New England Biolabs) digested with PstI and resulted after transformation in plasmid pLL62 (Fig. 6). The presence of the modified lacR gene in pLL62 was confirmed by digestions and PCR amplifications.

This plasmid was cotransformed with pLL55, pLL57 and pLL58 in E coli resulting in strains LZL63, LZL64 and LZL65 (Table I) respectively. The resulting strains contained the different L. delbrueckii promoters together with the LL44 lacR gene. Regulation of the promoters was tested following the chloramphenicol acetyltransferase assay of Shaw, supra in the presence of lactose or glucose. The results showed that after 4 min incubation at 37°C, strains containing the different promoters were fully induced in YT medium or in a minimal medium based on M9-salts in the presence or in the absence of lacR as shown in the table below :

Table I

chloramphenicol acetyltransferase assay (Shaw, 1975)

Plasmid	YT-broth		M9-salts	
	Sugar	OD_{415}	Sugar	OD_{415}
pKK232-8	glucose	1.8		
pKK232-8	lactose	1.9		
LZL63	glucose	2.6	glucose	0.7
N299 prom. (pLL55)	lactose	2.5	lactose	0.9
+ lacR (pLL62)				
LZL64	glucose	2.5	glucose	0.8
N141 prom. (pLL57)	lactose	0.4	lactose	1.2
+ lacR (pLL62)				
LZL65	glucose	1.2	glucose	1.3
LL44 prom. (pLL58)	lactose	1.1	lactose	1.0
+ lacR (pLL62)				

The OD_{415} was red following 4 min incubation at 37°C.

The same results were found for the Gram-positive bacterium Staphylococcus aureus, where the lac promoter was functional in E. coli but the lacR gene not (Oskouian et al. J. Bacteriol. **169** (1990), 5459-5465). One possible explanation is that an additional factor, present only in Gram-positive bacteria, is necessary for proper repressor function.

Example 5

Cloning of the L. delbrueckii lac promoters in front of the ß-glucuronidase gene in Lactococcus lactis

The lac promoters of LL44 (inducible) and N299 (constitutive) were cloned in front of a reporter gene, the ß-glucuronidase (gusA) gene. They were PCR amplified using primers SEQ ID No. 12 and No. 14, respectively, both containing an EcoRI site and primer SEQ ID No. 15 which is located in the lacS gene. The amplified fragments were digested with BspEI, located 170 bp downstream the lacS start codon, filled with T4 DNA polymerase, and digested with EcoRI. Fragments were then cloned in front of the promoterless gusA

gene of pNZ272 (Platteeuw et al, Appl. Env. Microb. **60** (1994), 587-593) digested with AvaII (refilled with T$_4$ DNA polymerase) and EcoRI. The resulting plasmids, pLL110 for LL44 and pLL113 for N299, were transformed in Lc. lactis (MG1363, plasmid free) (Fig. 7 and 8).

Chloramphenicol resistant clones were screened in the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-glu), which is cleaved by the glucuronidase generating a blue color due to the liberation of the indol group of X-glu. Both plasmids were able to express ß-glucuronidase, indicating that the lac promoter of L. delbrueckii is functional in Lc. lactis. The presence of the different promoters was checked by sequencing.

Example 6
Cloning of the L. delbrueckii lacR gene behind the ß-glucuronidase gene in Lactococcus lactis

To test if the lac promoters can be controlled by the L. delbrueckii lac repressor, the lacR gene of LL44 was cloned on plasmids pLL110 and pLL113. The lacR gene was PCR amplified using the following oligonucleotides:

<p style="text-align:center;">Xho I
ATAAAT<u>CTCGAG</u>TGGTGATTCAGCC   SEQ ID No. 20</p>

<p style="text-align:center;">Xho I
ATATTA<u>CTCGAG</u>ACAGAATGCAGCC   SEQ ID. No. 21</p>

both containing a XhoI site. The primers were chosen to induce flanking regions of the gene. That is the oligonucleotide SEQ ID No. 20 is located 80 bp upstream the lacR start codon in the lacZ gene and the oligonucleotide SEQ ID No. 21 70 bp downstream the lacR stop codon, in the Asn-tRNA synthetase (asnA) gene. The fragment was cleaved with XhoI and cloned in the same site downstream the gusA gene of pLL110 and pLL113. With pLL110 containing the LL44 lac promoter, one clone (pLL112) was obtained with a lacR gene inserted in the opposite (rev) orientation as the gusA gene (Fig. 7). With pLL113 containing the N299 promoter, the lacR gene could be cloned in both orientations, pLL115 in the same (for) and pLL116 in the reverse (rev) orientation as the gusA gene (Fig. 8). The

mutated lac repressor (⊗lacR) gene of LZL102 was PCR amplified and cloned as described above for LL44, resulting in plasmids pLL111 with the lac promoter of LL44 and pLL114 with the promoter of N299, both in the same orientation as gusA.

Example 7
Control of the ß-glucuronidase activity in Lactococcus lactis

The different constructs containing the L. delbrueckii lac promoters and repressors were studied by growing the transformed Lc. lactis in the presence of mannose. This sugar was shown to have no influence on the lactose metabolism of Lactococcus, as compared to saccharose and cellobiose. Exponentially growing cells were diluted into fresh media containing different concentrations of lactose and glucose, grown and harvested. ß-glucuronidase (GusA) activity was measured from permeabilized cells in the presence of p-nitrophenol-β-D-glucuronide, which is cleaved by GusA, liberating para-nitrophenol staining the solution in yellow. The results of these experiments are shown in figure 9.

Example 8
Regulation of the lac promoter of L. delbrueckii subsp. lactis LL44

The promoter of LL44 (pLL110) was functional in Lc. lactis resulting in the production of ß-glucuronidase in the presence of lactose or mannose. When glucose was added to the medium with or without lactose, a five to seven fold reduction in GusA activity was observed. This indicates that the glucose catabolite repression is efficient in the system.

Table II

Expression of the gusA gene fused to the L. delbrueckii lac promotor

in Lactococcus lactis MG1363 grown in M7

Beta-glucuronidase activity (U/mg prot. x 10^{-4})$^{\alpha}$

Sugar added	L. lactis LL44 promoter				L. bulgaricus N299 promoter		
	pLL110 -	pLL111 +⊗lacR[b]	pLL112 +lacR	pLL113 -	pLL114 +⊗lacR	pLL115 +lacR	pLL116 +lacR
0.5% mannose	20.1±8.0	35.7±9.8	0.5±0.5	35.8±0.5	9.9±0.6	1.1±1.2	0.7±0.7
0.05% lactose	15.6±4.4	35.8±7.1	3.4±1.1	32.0±4.1	10.5±2.2	0.7±0.7	0.7±0.8
0.2% lactose	15.1±4.5	34.1±7.5	20.7±13.4	34.4±7.2	8.2±1.6	0.9±1.2	0.3±0.1
0.5% lactose	20.1±12.6	37.0±7.8	27.1±7.5	34.1±9.4	8.5±1.1	1.5±1.0	11.1±1.3
1.0% lactose	15.3±6.0	29.7±1.3	23.8±8.6	33.7±7.0	8.7±0.8	3.2±0.5	12.8±4.8
0.5% glucose	5.1±3.7	34.6±9.1	0.5±0.2	22.3±2.0	5.6±1.0	0.7±0.8	0.6±0.6
0.5% glucose + 0.5% lactose	3.5±1.4	32.5±4.9	3.3±1.6	28.0±7.6	6.0±0.8	0.6±0.6	2.0±0.7
0.5% glucose + 1.0% lactose	3.7±0.9	38.7±12.0	4.8±2.2	22.5±6.6	4.7±1.0	0.6±0.2	4.0±1.2

In the presence of the repressor gene (pLL112), GusA activities were similar to those observed with pLL110 when lactose (0.2 to 1%) was added. Almost no activity was observed with mannose (Table X) indicating that the repressor is expressed in Lc. lactis and can bind to the lac promoter. Galactose, which is a common inducer of the lac system showed no induction of the system. Plasmid pLL112 was also subjected to the glucose catabolite repression, with a five to nine fold reduction in activity.

Surprising results were obtained with pLL111, the plasmid which contains the ⊗lacR of LZL1012 and the LL44 lac promoter. GusA activities were twice higher than with the lac promoter alone even in the presence of glucose (Table II). The mutated repressor, with half of the protein translated, seems to play the role of an enhancer.

Example 9
Regulation of the lac promoter of L. delbrueckii subsp. bulgaricus N299

The lac promoter of N299 (pLL113) induced production of GusA activity, almost twice as much as that produced by the promoter of LL44 (Table II, above). The N299 promoter is thus also functional in Lc. lactis. In the presence of glucose, only a relatively weak repression was observed, which could be explained by a different sequence of the CRE motif.

In the presence of the LL44 lacR gene, cloned in both orientations behind the gusA gene (pLL115, for and pLL116, rev), almost no GusA activity was detected in the presence of mannose. This result indicates that the so called "constitutive" promoter of L. delbrueckii subsp. bulgaricus is in fact repressed by the L. delbrueckii lac repressor. In addition, increasing concentrations of lactose induced increasing production of GusA activity. Nevertheless, the production even at 1% lactose with the repressor in the reverse orientation (pLL116) reached only half of that obtained in the absence of repressor. The N299 is not only regulated by the repressor, but more tightly than that of LL44 (Table V).

Finally in the presence of the mutated repressor (pLL114), the GusA activity was 3 to 4 times lower than in the absence of any repressor. On this promoter the ⊗lacR seems to have a general inhibiting activity as a low activity is observed with or without lactose.

Example 10

Lactose induction of L. delbrueckii promoters in Lactococcus lactis

The GusA activity was measured at several times during exponential growth of Lc. lactis containing the lac promoters of LL44 and N299 and the lacR gene of LL44 (pLL112, pLL115 and pLL116) in the presence of different concentrations of lactose and glucose (Fig. 9). The lac promoter of LL44 was maximally induced with 1% lactose and clearly subjected to glucose catabolite repression, i.e. 0.5% glucose in addition to lactose reduced this activity by half. As expected, mannose and glucose alone generated no GusA activity. Plasmids containing the N299 lac promoter (pLL115, for and pLL116, rev) are hardly inducible and are completely inhibited by glucose. Plasmid pLL115, having the repressor gene in the same orientation as gusA, resulted in the lowest lactose induction. In this case, it can be postulated that the repressor is produced in large amounts under the control of the L. delbrueckii lac promoter whereas in pLL116, it must be under the control of a weak promoter present on pNZ273 and is thus produced in smaller amounts.

Example 11

ß-galactosidase activity in Lactobacillus delbrueckii

The relation between the lac promoter and the lac repressor were studied in situ. ß-galacto-sidase (β-gal) activity was measured in different L. delbrueckii strains in BHI-broth containing different sugars. The L. delbrueckii subsp. lactis strains tested showed no or low β-gal activity in the presence of mannose/galactose or glucose

Table III

Expression of the lacZ gene in different strains of
Lactobacillus delbrueckii grown in BHI

Sugar added	Beta-galactosidase activity (mU/OD600						
	LL44	LZL102	LB68	N62	N141	LB10	N299
0.5% galactose	2.4/1.8	6.4/9.5	2.4/2.0	0.0/0.0	0.6/0.5^a	15.3/12.6	7.9/14.9
0.02% lactose	14.1/17.5	12.1/9.5	21.2/20.6	11.5/9.5	13.5/9.7	18.0/12.1	14.3/10.6
0.05% lactose	11.4/13.9	7.4/9.5	24.3/20.6	11.6/9.1	11.0/10.9	16.9/8.6	9.5/18.1
0.2% lactose	10.1/12.3	11.6/9.5	14.1/20.3	8.8/8.1	7.6/8.3	10.2/7.4	23.8/23.8
0.5% lactose	19.0/8.7	10.6/9.5	20.4/16.5	15.4/12.4	9.8/5.5	12.8/11.1	29.0
0.5% glucose	0.9/0.6	14.7/9.5	3.2/1.9	0.0/0.0	2.5/2.2	12.6/7.8	39.7/15.5
0.5% glucose + 0.5% lactose	15.9/7.1	11.7/9.5	15.6/16.8	17.9/33.1	6.3/5.4	8.0/7.6	28.6/31.7
0.5% glucose + 1.0% lactose	7.7/6.7	16.6/9.5	15.4/15.5	14.9/13.1	9.3/7.0	12.0/10.3	22.0/23.8

a : galactose was replaced by mannose

At all concentrations of lactose tested (0.02 to 0.5%), the β-gal activity was in the same range, indicating that very low concentration of lactose is able to fully induce the expression of the lac operon. In the presence of lactose and glucose, strains were not subjected to catabolite repression and even a stimulation of β-gal activity was found for N62 (Table III, above). The constitutive L. delbrueckii subsp. bulgaricus N299 showed the same β-gal activity with all sugars used even in the presence of glucose. Two mutants of L. delbrueckii subsp. lactis, LB10 and LZL102, were also analysed. LZL102, the spontaneous mutant of LL44, produces a truncated repressor and LB10 produces no peptide at all (Fig. 12). In both cases, strains were constitutive with equal amounts of β-gal produced with galactose, lactose or glucose.

Example 12

Lactose induction of the lac promoters in Lactobacillus delbrueckii

The β-gal induction was measured at several times during exponential growth of the L. delbrueckii subsp. lactis strain LL44 and the bulgaricus strain N299. The maximum level of activity was reached in both strains after 15 min incubation (Fig. 10). For LL44, no β-gal activity was detected with galactose or glucose, whereas full induction was obtained with all lactose concentrations. The enzymatic activity was slightly reduced in the presence of glucose, i.e. a full activity was only reached after 60 min. For N299, all sugars resulted in the same induction with no glucose catabolite repression.